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RESEARCH ARTICLE

Increase of calcium and reduction of lactose concentration in milk by treatment with kefir grains and eggshell

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ABSTRACT

Dairy products are the main source of calcium (Ca), but the loss of the consumption habit contributes to low consumption in adulthood, which leads to osteoporosis and increased fracture risk. Domestic use of kefir is straightforward and the eggshell is a natural discarded source of Ca. This paper proposes the development of an enriched Ca reduced lactose milk using eggshell and kefir. During the *in vitro* preparation, the pH, Ca and lactose contents were measured. Ca intestinal absorption of untreated milk and milk with kefir was compared. Finally, human volunteers consumed this dairy product and 24-h urine Ca was measured. Results showed that the beverage has lower lactose and higher Ca than untreated milk and milk with kefir. Intestinal Ca absorption was not different between both milks and an increase in urinary Ca excretion was observed in humans. This study provides a methodology to prepare at home a dairy product that could contribute to improve the Ca intake in adults.

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KEYWORDS
Calcium; dairy milk; eggshell; kefir grains

Introduction

Calcium (Ca) is an essential mineral not only for calcified tissue formation but also for excitability and permeability of the plasma membrane, exocrine secretion, enzyme regulation, neurotransmission, muscle contraction, and signal transduction pathways (Rasmussen, 1986a,b).

Dietary sources and Ca intake have been modified during man evolution. Primitive man got Ca from roots, tubers, nuts, and legumes in quantities that are supposed exceeded 1500 mg/day and may be increased to twice in the hunter-gatherer period (Torresani, 2007). The modern human consumes a smaller amount of Ca and dairy products are currently providing the most amount of Ca in the diet. In turn, the Committee on Nutrition of the United States concluded that there is a low Ca intake by more than 50% of children aged 1–5 years (Greer & Krebs, 2006). Other study reveals that a high percentage of the world population does not cover the daily requirement of Ca (Bailey et al., 2010). In Argentina, the insufficient Ca intake reached 6–21% in children under 2 years (Ministerio de Salud, 2010) and 28–49% in older children (Ministerio de Salud, 2012). The importance of Ca intake was demonstrated in epidemiological studies with women of 20–49 years old which indicates that milk consumption during childhood and adolescence is positively correlated with bone mass peak (Kalkwarf et al., 2003; Wosje & Specker, 2000).

One of the major cause of the decrease in Ca intake is the decrease in consumption of milk because of digestive problems due to "no persistence of lactasa or hypolactasia" present in high percentage of world population (Lomer et al., 2008; Wang et al., 1998). Usually this pathology is self-diagnosed (Nicklas et al., 2011) leading to the suspension of dairy products consumption, which leads to decreased bone mineral density and increased fracture risk (Di Stefano et al., 2002). It is well documented that the use of fermented milk such as yogurt, is better tolerated by people with hypolactasia (Onwulata et al., 1989) and Ca absorption is higher in milk with hydrolyzed lactose than milk without lactose (Nielsen et al., 1984). Kefir milk is one of the fermented milk produced by lactic acid and carbon dioxide producing microorganisms (*Lactococcus lactis*, *Lactobacillus kefir*, *Lactobacillus plantarum*, *Acetobacter*, *Saccharomyces*, among others) known as kefir grains (Garrote et al., 2001). It is proved that kefir consumption significantly reduces lactose intolerance (Hertzler & Clancy, 2003) and also produces lower prevalence of caries (Tanaka et al., 2010), it does not

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promote tooth enamel demineralization (Lodi et al., 2010), improved lipid profile as increased HDL cholesterol and lowered LDL/HDL cholesterol ratio (Sadrzadeh-Yeganeh et al., 2010). A recent work also demonstrated that kefir could improve bone mass and the biomechanical properties of bone (Chen et al., 2015).

The preparation of milk with kefir is easy and safe and can be prepared from different types of milk. The stability of the grains is high; whether they are stored at room temperature or common refrigerator (Magalhães et al., 2010; Witthuhn et al., 2005). The mass of kefir grains increases after each fermentation being little affected by environmental or chemical conditions (Guzel-Seydim et al., 2011). The arrangement of the microorganisms in the grain has a great antimicrobial power (Ismaiel et al., 2011).

A liter of milk provides the amount of Ca necessary for the daily requirement of an adult, because milk has Ca content between 100 and 140 mg/100 ml. With a Ca fortified milk with 200 mg Ca/100 ml the milk consumption would be reduced by half. A recently published work from our laboratory showed that an eggshell contains about 2 g of Ca with the same bioavailability as Ca carbonate (Brun et al., 2013). To achieve this result, about 2 g of eggshell/day/person would be enough. Therefore, the use of eggshell as a way to enrich foods with Ca would have economic benefits particularly for low-income individuals.

This study proposes a non-pharmacological way of improving Ca intake by the development of a dairy product with increased Ca content and reduced lactose using kefir and eggshell.

Materials and methods

All determinations were carried out following standard operating protocols. The measurements were carried out under quality control, rejecting the values if the coefficient of variation exceeded 10%. Simultaneously quality control solutions of known concentration were processed, if the standard deviation units were outside the range [−2, 2], the measurement of the entire batch of samples was repeated.

In vitro experiments

pH, lactose, and Ca content determination

The values of pH as well as the lactose and Ca contents of milk with different treatments were measured. The following experimental treatments were done:

M: 40 ml of milk without treatment (control).

MCa: 40 ml of milk with the addition of 200 mg of powdered eggshell as source of Ca.

MK: 40 ml of milk with the addition of 2.5 g of kefir grains.

MKCa: 40 ml of milk with the addition of 200 mg of powdered eggshell and 2.5 g of kefir grains.

The samples were incubated for 34 h at room temperature (23 ± 3 °C). Samples were taken at the beginning of the experiment (baseline, 0), 120, 480, 600, 730, 945, 1065, and 1185 min for lactose and Ca measurements. pH was measured at 0, 730, 945, 1065, and 1185 min. This experiment was repeated three times.

Commercial skimmed milk with 110 mg/100 ml of Ca and desiccated kefir (Prama®, Argentina) were used in the experiments described in this paper. Dry eggshells were powdered using a rolling pin and after that a small sieve was used to separate the particles of larger size as was described previously (Brun et al., 2013). The eggshell powder was sterilized in an automatic sterilizer autoclave (Microclave SL 9000, Buenos Aires, Argentina) for 15 min at 134 °C and it was dried in an oven at 37 °C for 30 min to avoid Salmonella contamination.

Lactose, Ca, and fat content in MKCa prepared in a domestic form

MKCa was prepared in a domestic form as follows: 500 ml of skimmed milk were maintained for 24 h in a refrigerator (8 °C) with the addition of 50 g of kefir grains. Afterwards, the treated milk was sieved and was transferred to another vessel where two eggshells, previously boiled in water for 5 min, were added and then completed up to 1 l with milk. This vessel remained in the refrigerator (8 °C). After 24 h, 1 ml sample was obtained and treated with 50 µl of concentrated HCl in order to precipitate the proteins. The samples ($n = 22$) were centrifuged at 8500 rpm and the supernatant was used to measure the concentration of lactose and Ca. MKCa was prepared daily for one month and daily samples were collected and the values of Ca and lactose concentration were compared with untreated milk (M). In addition, total fat ($n = 4$) and triglycerides ($n = 16$) were measured as describe below.

In vivo *experiments*

In situ isolated duodenal loops in rats

This experiment was carried out to assess whether kefir affects or not Ca absorption. Adult Sprague-Dawley rats were anesthetized intraperitoneally with urethane (120 mg/100 g body weight) and kept in thermostated stretchers. During the experiment, room temperature was kept between 21 and 22 °C and rats body temperature was kept at 35 ± 1 °C with an infrared lamp.

A 5-cm portion from the distal duodenum to the pylorus was isolated (Brun et al., 2012) and a catheter was placed at the distal end. A volume of 1 ml of filling solution was introduced in the *in situ* isolated duodenum loops through the catheter. The filling solution of the control group ($n = 8$) consisted of untreated milk (M) whereas in the treated group ($n = 8$) the dairy product made with milk and 2.5 g of kefir grains (MK) was used.

Samples were obtained immediately after filling and after 30 min of incubation for the measurement of Ca concentration.

The rats were treated according to the accepted international standards for animal care (Olfert et al., 1993). This work has been approved by the Ethical Committee of the School of Medicine of Rosario National University.

Ca bioavailability in humans' volunteers

The Ca bioavailability was assessed in 17 healthy volunteers. Volunteers were members of the laboratory between 25 and 50 years of age without history of hypolactasia or Ca metabolism disorders. A randomized crossed over study was carried out. The volunteers ($n = 12$) were randomized to drink untreated milk (M) or milk treated with kefir and eggshell (MKCa) prepared in a domestic form as described previously. In addition, volunteers ($n = 5$) were randomized to drink milk treated with kefir (MK) as control feeding or milk treated with kefir and eggshell (MKCa). After 7 d, they were crossed over onto the alternative treatment.

The basal background Ca intake was calculated (490 ± 235 mgCa/24 h) and remained constant through the experiment (Friedman test, $p > 0.05$). In addition similar conditions of physical activity, hydration, type, and amount of food eaten remained constant through the experiment. Therefore, the only significant change was the difference in Ca content between M or MK and MKCa. The hypothesis was that with MKCa consumption, more Ca is absorbed than with untreated milk or milk treated with kefir. As the body is in Ca balance, there should be more urinary Ca excretion when MKCa is used compared to untreated milk or milk treated with kefir (assuming no increase in the fecal excretion). Each volunteer ingested 1 liter of milk (control day, M) with 94.1 ± 8.6 mg/100 ml of Ca or milk treated with kefir (control day, MK) with 84.1 ± 22.8 mg/100 ml of Ca, divided into four doses of 0.25 liter: at 8 am, 12 am, 4 pm, and 10 pm. Urine was collected for 24 h for Ca measurement (mg Ca/24 h). Urine collection was performed as follows: at 8 am urine was issued and discarded, and from this time urine was collected in a container until 8 am of the next day. The urine was collected with a drop of concentrated HCl to avoid precipitation of Ca phosphate and to prevent bacterial growth. The same experiment was repeated after 7 d with the MKCa consumption with 162.2 ± 42.1/100 ml of Ca.

In addition, MK and MKCa were evaluated in order to know the volunteers acceptability, which was subjectively evaluated by volunteers using a quiz.

This study was conducted according to the guidelines laid down in the declaration of Helsinki and all procedures were supervised by a physician. Informed consent was witnessed and formally recorded.

pH measurement

The pH was measured immediately after the sample was extracted using a Methrom 632 pH meter calibrated for the range of 4–7.

Lactose measurement

As lactose is formed by one glucose and one galactose, the lactose concentration was measured using the glucose determination after acid hydrolysis using a commercial kit (Wiener, Rosario, Argentina). The kit does not detect galactose and/or lactose. Briefly, 1000 µl of the supernatant obtained after precipitation with HCl and subsequent centrifugation were taken. About 50 µl of concentrated HCl were added and incubated for 30 min at 100 °C to hydrolyze lactose into glucose and galactose. Finally, the glucose concentration (g/100 ml) was measured in 5 µl of the sample.

The glucose content of untreated milk, as well as the treated milk with kefir and/or eggshell was under the detection limit of the technique used to measure glucose. The lactose content in the samples was calculated using the following equation, taking into account the molecular weight of glucose (180 Da) and lactose (342 Da): lactose (g/100 ml) = glucose (g/100 ml) × 342/180.

Calcium measurement

Ca was measured by atomic absorption spectrometry (Arolab MK II, Buenos Aires, Argentina). The samples were diluted and strontium chloride was added to obtain a final concentration of 2 g/100 ml. Strontium chloride was added to remove interference by Ca complexing anions. About 1 ml of the sample was volatilized in an acetylene–oxygen flame and the absorbance at 4240 nm from a Ca lamp was measured. The Ca concentration in the sample was determined by comparison with standard solutions of Ca (1–100 µg/ml) processed simultaneously and in the same way.

Total fat and triglycerides measurement

Total fat was determined by Rose-Gottlieb method (Rose-Gottlieb Reference Method, 1973) and triglycerides was measured with a commercial kit (TG color, Wiener Lab, Rosario, Argentina).

Statistical analyses

Data were analyzed with R 2.14.0 (R Development Core Team, 2011) and were expressed as mean ± SD. When treatments were compared along time two-way ANOVA was used, and differences among means were compared with multiple comparison test LSD. Urinary Ca excretions in human volunteers after M or MK and MKCa consumption were compared using Student's *t*-test for paired data. Significant differences were considered when $p < 0.05$.

Results

In vitro experiments

pH, lactose, and Ca content determination

After 730 min, a significant decrease in pH was observed in MK and MKCa groups compared to M and MCa groups. M and MCa treatments maintained the pH value without changes over time (Figure 1).

A significant decrease in lactose content was observed over time in the groups containing kefir grains, MK, and MKCa (Figure 2). In turn, a significant decrease in the lactose content of MK and MKCa groups compared to controls (M and MCa) was observed. Finally, the M and MCa groups showed no significant changes in the content of lactose over time. The lactose content decreased by 12.7 ± 2.49% for MK group and 15.9 ± 3.28% for MKCa group.

Finally, no significant difference in Ca concentration over time between M, MCa, and MK groups was observed. However, after 945 min the MKCa group significantly increased its Ca content compared to the other groups (Figure 3). The Ca content increased by 42.1 ± 16.18% at the end of treatment in MKCa group.

Lactose, Ca, and fat content in MKCa prepared in a domestic form

No correlation was found between the lactose content and the Ca content in MKCa samples. However, lactose content in MKCa group was lower in all the samples than in untreated milk (M) and the Ca content in milk with kefir and eggshell (MKCa) was equal or greater (Figure 4).

Neither triglycerides content nor total fat content were different between milk and milk treated with kefir and eggshell (triglycerides = M: 1.83 ± 0.54 g/100 ml; MKCa 1.83 ± 0.31 g/100 ml; paired *t*-test, $p > 0.05$. Total fat = M: 2.8 ± 0.05 g/100 ml; MKCa: 2.9 ± 0.03 g/100 ml; paired *t*-test, $p > 0.05$).

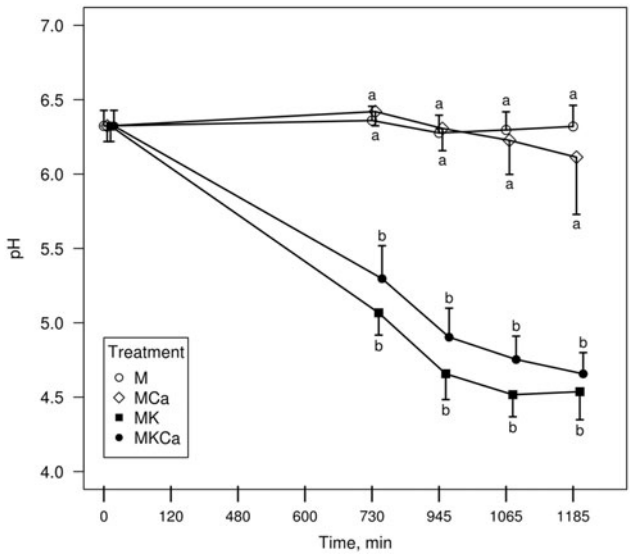

Figure 1. pH values of experimental groups (M, MCa, MK, and MKCa) over time. Values are shown as mean ± SD of three experiments. All the letters different between points indicate significant differences between them. Only statistically differences are shown in the figure (two-way ANOVA, LSD post-test, $p < 0.05$).

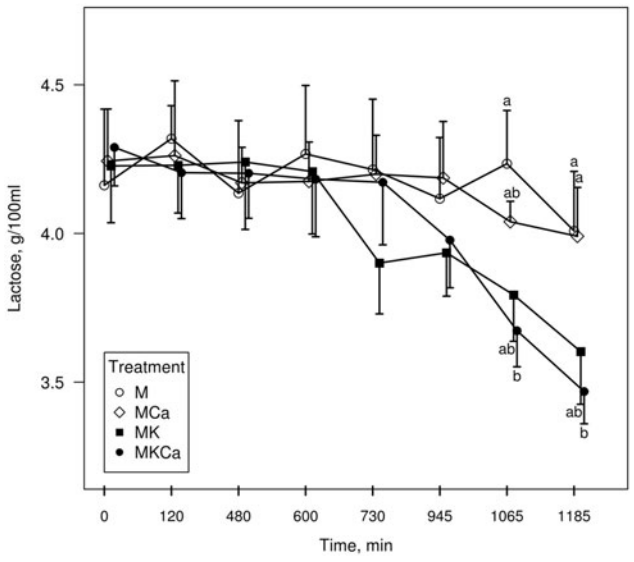

Figure 2. Lactose content (g/100 ml) of experimental groups (M, MCa, MK, and MKCa) over time. Values are shown as mean ± SD of three experiments. All the letters different between points indicate significant differences between them. Only statistically differences are shown in the figure (two-way ANOVA, LSD post-test, $p < 0.05$).

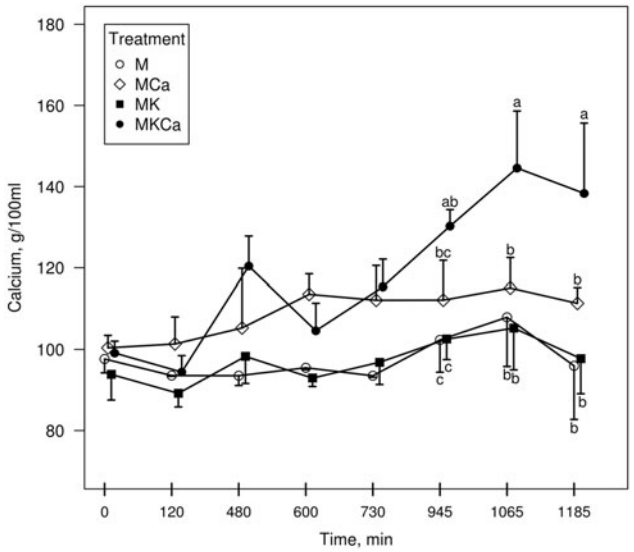

Figure 3. Ca content (g/100 ml) of experimental groups (M, MCa, MK, and MKCa) over time. Values are shown as mean ± SD of three experiments. All the letters different between points indicate significant differences between them. Only statistically differences are shown in the figure (two-way ANOVA, LSD post-test, $p < 0.05$).

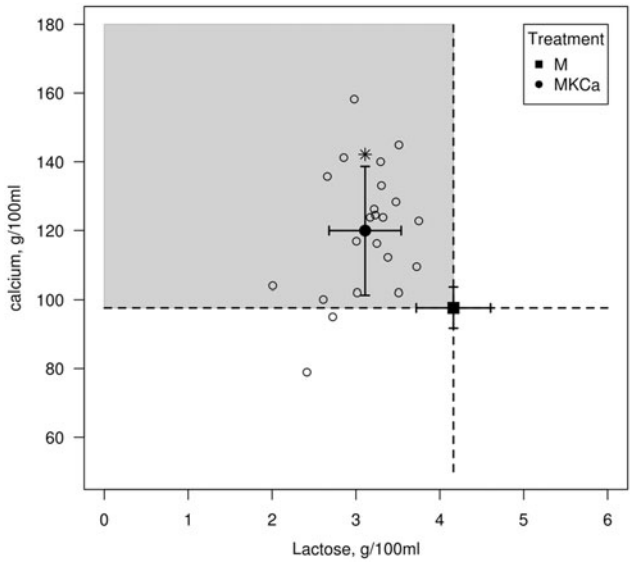

Figure 4. Lactose and Ca contents (g/100 ml) of the MKCa and M. The dairy milk was prepared daily for one month and daily samples were collected. Black symbols are the mean ± SD of lactose and calcium contents for M and MKCa. The dashed lines indicate the mean of Ca and lactose contents of untreated milk (M) and white points show all the values measured of the dairy product prepared with kefir and eggshell (MKCa). The gray rectangle shows the part of the figure where low lactose and high Ca values can be seen. *Indicates significant differences of both Ca and lactose contents between M and MKCa groups (Student's *t*-test, $p < 0.05$).

In vivo *experiments*

A significant decrease in Ca concentration after 30 min in the intestinal isolated loop was observed for both untreated milk (M) and milk treated with kefir (MK) groups. However, duodenal Ca absorption in rats treated with M was not different from rats treated with MK product (Table 1).

Table 1. Ca concentration (g/100 ml) before and after 30 min incubation of untreated milk (M) or milk with kefir (MK) in *in situ* isolated duodenal loops experiment.

Time (min)	M	MK
0	86.9 ± 3.23^{a}	91.2 ± 3.29^{a}
30	66.5 ± 5.70^{b}	63.4 ± 6.29^{b}

Values are shown as mean ± SD. All the letters different between groups indicate significant differences between them (two-way ANOVA, LSD post-test, $p < 0.05$).

Finally, 24-h urinary Ca excretion in human volunteers ($n = 12$) was significantly lower when untreated milk (M) with 94.1 ± 8.6 mg/100 ml of Ca was consumed than when the same volume of milk treated with kefir and eggshell with was consumed (MKCa) (Figure 5, panel A). Similarly, urinary Ca excretion in volunteers ($n = 5$) was significantly lower when milk treated with kefir (MK) with 84.1 ± 22.8 mg/100 ml of Ca was consumed than when the same volume of milk treated with kefir and eggshell was consumed (MKCa) (Figure 5, panel B). The Ca concentration of MKCa was 162.2 ± 42.1 mg/100 ml and was significantly higher compared with M or MK (Student's *t*-test, $p < 0.05$).

MK and MKCa palatability was evaluated in volunteers to study their acceptability. For the 83% of volunteers, there were no differences between MK and MKCa. The other 17% described MKCa as more acidic than MK. No volunteer identified eggshell in MKCa. For the 23.1% MK and MKCa acceptability was very good, 15.4% described as acceptable and 61.5% must add an additive such as fruits. These last groups described MK and MKCa as very acidic (83%) or with sour milk taste (17%).

Finally, 53.8% of volunteers considered that MKCa is a good resource to increase Ca intake, 15.4% considered that even not taking good taste they would consume it knowing its properties, and 30.8% considered that its use is not justified having other sources of Ca.

Discussion

The non-persistence of intestinal lactase or hypolactasia affects a large percentage of the population, leading to a decrease in milk consumption habit and thus a deficiency in Ca intake. It is particularly important in children because of the Ca requirement for growth and the formation of peak bone mass and in postmenopausal women who have an accelerated loss of bone Ca by estrogen deficiency. A solution to this it would be the use of lactose-reduced milk or Ca carbonate tablets. However,

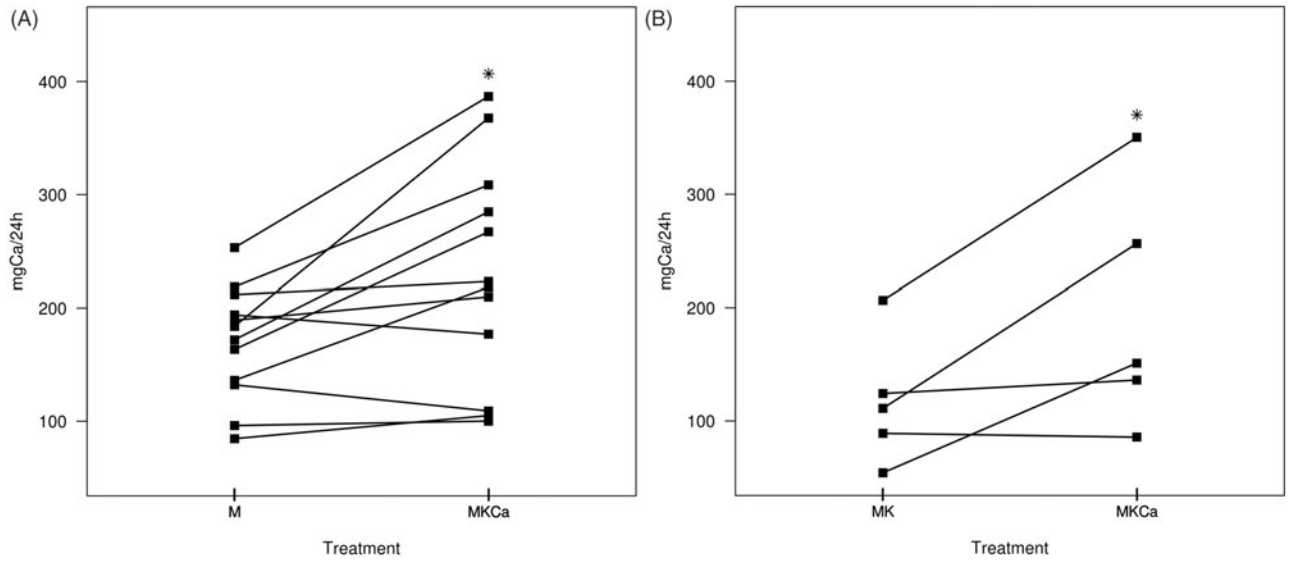

Figure 5. Panel A. Urinary Ca excretion (mg Ca/24 h) of volunteers ($n = 12$) that consumed untreated milk (M) and milk with kefir and eggshell (MKCa). Panel B. Urinary Ca excretion (mg Ca/24 h) of volunteers ($n = 5$) that consumed milk treated with kefir (MK) and milk with kefir and eggshell (MKCa). *Indicates significant differences of calcium excretion between MKCa and M or MK groups (Student's *t*-test for paired data, $p < 0.05$).

these treatments are costly and supplementation with tablets sometimes involves difficulties of adherence to treatment.

It is proved that kefir consumption significantly reduced lactose intolerance (Onwulata et al., 1989) increasing consumption of dairy products. Additionally, consumption of fermented milk products such as yogurt or kefir is better accepted among people with hypolactasia (Hertzler & Clancy, 2003).

Since hypolactasia generates a lower Ca intake we proposed a domestic procedure to obtain milk easily prepared at home with increased Ca content and reduced lactose content. In order to obtain a high Ca content we used eggshell as source of Ca and kefir grains to reduce lactose content.

Both MK and MKCa milk significantly reduced their pH compared to M and MCa groups. The lower pH value greatly facilitated the eggshell dissolution (Greer & Krebs, 2006) as Ca carbonate is the major component of the eggshell and this salt is soluble in an acid medium. The equilibrium of Ca carbonate in acidic medium has an overall constant of 1.3×10^{11} and at pH lower than 6, the theoretical concentration of ionized Ca is higher than 130 g/l. As the pH obtained with the dairy MKCa ranges between 4 and 5.5, the solubility of the eggshell (Ca carbonate) is almost total. Therefore, an increase of 42.1% in Ca content was obtained in MKCa milk with a reduction of 15.9% in lactose content. MK also reduced the lactose content (12.7%) but without the increased Ca content. This Ca increase in MKCa dairy product is important for those individuals who have a decrease in dairy products consumption due to hypolactasia. As the Ca content increased 2-fold, it is required to drink half of the milk to consume the recommended daily Ca content. Although the reduced lactose content could promote milk consumption in patients with hypolactasia, this was not assessed in our work. Even though the lactose content was significantly reduced (3.5 ± 0.11 g/100 ml), the absolute value is greater than the lactose-reduced milk (0.3–0.9 g/100 ml) that are available for these patients.

In order to determine whether the kefir grains and decrease in pH can affect the Ca absorption, *in situ* isolated duodenal loops in rats were performed. Absorption of untreated milk (M) and milk treated with kefir (MK), both with the same concentration of Ca, was compared. The results showed that kefir did not impair intestinal Ca absorption compared with untreated milk. On the other hand, after 30 min of treatment, a significant Ca absorption was observed. Therefore, Ca could be absorbed in the same way from milk treated with kefir grains.

As limitation of the study we can mention that performing a proper Ca balance experiment requires more accurate methods that a simple analysis of urinary Ca excretion, however the *in vivo* experiment in young healthy volunteers without history of hypolactasia or Ca metabolism disorders could suggest that greater urinary Ca excretion is a consequence of an increase in Ca absorption because of MKCa product ingestion. Moreover, in a previous paper (Chen et al., 2015) kefir treatment in ovariectomized rats significantly decreased the levels of the carboxyl-terminal cross-linking telopeptides of type I collagen, a bone resorption marker and

prevented bone loss. Therefore, the increase in urine Ca observed in our study would not be caused by an increase in bone resorption.

In summary, a procedure to prepare a dairy product with increased Ca content and reduced lactose was developed. Kefir grains to ferment milk and reduce lactose content were used and the eggshell was used to increase Ca content. Intestinal Ca absorption was not different between untreated milk and milk treated with kefir grains. Finally, humans that consumed this dairy product had an increased urinary Ca excretion with variable acceptability: while 38% of volunteers considered MKCa acceptable, the other 61.5% considered it not acceptable. However, 69.2% considered that MKCa product is a good resource to increase Ca intake even not taking good taste and 30.8% considered that its use is not justified having other sources of Ca. Although the acceptability of the dairy MKCa varied between volunteers, the majority of them considered it a good resource for increasing Ca intake. In addition, MKCa milk is easy to prepare and very cheap, since the kefir grains are reusable and eggshell is a household waste. Therefore, this product is very affordable for people with low income.

Disclosure statement

Brenda Lorena Fina, Lucas Ricardo Brun, and Alfredo Rigalli declare that they have no conflict of interest.

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